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TITLE: MELANOCORTIN-4 RECEPTOR GENE AND USE AS A GENETIC MARKER FOR FAT CONTENT, WEIGHT GAIN, AND/OR FEED CONSUMPTION OF ANIMALS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/094,287 filed July 27, 1998 and U.S. Provisional Application Serial No. 60/116,186, filed January 15, 1999, the disclosures of which are hereby incorporated by reference.

GRANT REFERENCE CLAUSE

This invention was supported at least in part by grants from the United States Department of Agriculture through the Iowa Agriculture and Home Economics Experiment Station (IaHees) and Project Number IOW03148 (Hatch Funds). The United States government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to a method of genetically evaluating animals by assaying for the presence of at least one genetic marker which is indicative of one or more of the traits of fat content, growth rate, and feed consumption. In particular, the method analyzes for variation in the melanocortin-4 receptor (MC4R) gene which is indicative of these traits. Even more particularly, the method analyzes for a polymorphism in the MC4R gene.

BACKGROUND OF THE INVENTION

There is an increasing consumer demand for meat products having low fat content. This demand is fueled by accumulating evidence in the scientific literature that a high consumption of animal fat, especially fat with a high proportion of saturated fatty acids, represents a significant health hazard, including risk for cardiovascular disease. Other health concerns associated with high fat meats include their high content of cholesterol and the addition of relatively high amounts of salt which are added to improve the binding characteristics since salt aids in extracting the native water binding component myosin from the meat. Furthermore, an increasing number of

consumers find meat products containing chemical additives such as phosphates, emulsifying additives, and anti-oxidants less acceptable.

Faced with consumers who seek a healthier meat product, meat producers are continually pressed to offer cheaper and healthier products.

Cheaper products, of course, come from lowering costs of production. Producers are always interested in improving the growth rate and feed conversion of their animals. Lower production costs come from the shorter time to market and lower costs of feeding an animal. This can increase the profit margin in the livestock industry and/or result in lower prices to the consumer.

By being able to select for animals which have the aforementioned traits, producers can raise animals with these desirable characteristics. Selection for desirable traits has traditionally been done using breeding techniques.

Genetic differences exist among individual meat producing animals as well as among breeds which can be exploited by breeding techniques to achieve animals with these desirable characteristics. For example, Chinese breeds are known for reaching puberty at an early age and for their large litter size, while American breeds are known for their greater growth rates and leanness. Thus, it would be desirable to combine the best characteristics of both types of these breeds, thereby improving pork production.

Often, however, heritability for desired traits is low, for example, heritability for litter size is around 10%-15%. Standard breeding methods which select individuals based upon phenotypic variations do not take fully into account genetic variability or complex gene interactions which exist. Therefore, there is a need for an approach that deals with selection for leanness, growth rate, and feed consumption at the cellular or DNA level. This method will provide a method for genetically evaluating animals to enable breeders to more accurately select those animals which not only phenotypically express desirable traits but those which express favorable underlying genetic criteria. This has largely been accomplished to date by marker assisted selection.

Restriction fragment length polymorphism (RFLP) analysis has been used by several groups to study pig DNA. Jung et al., Theor. Appl. Genet., 77:271-274 (1989), incorporated herein by reference, discloses the use of RFLP techniques to show genetic variability between two pig breeds. Polymorphism was demonstrated for swine leukocyte antigen (SLA) Class I genes in these breeds. Hoganson et al., Abstract for Annual Meeting

of Midwestern Section of the American Society of Animal Science, March 26-28, 1990, incorporated herein by reference, reports on the polymorphism of swine major histocompatibility complex (MHC) genes for Chinese pigs, also demonstrated by RFLP analysis. Jung et al., Animal Genetics, 26:79-91 (1989), incorporated herein by reference, reports on RFLP analysis of SLA Class I genes in certain boars. The authors state that the results suggest that there may be an association between swine SLA/MHC Class I genes and production and performance traits. They further state that the use of SLA Class I restriction fragments, as genetic markers, may have potential in the future for improving pig growth performance.

The ability to follow a specific favorable genetic allele involves a novel and lengthy process of the identification of a DNA molecular marker for a major effect gene. The marker may be linked to a single gene with a major effect or linked to a number of genes with additive effects. DNA markers have several advantages; segregation is easy to measure and is unambiguous, and DNA markers are co-dominant, i.e., heterozygous and homozygous animals can be distinctively identified. Once a marker system is established selection decisions could be made very easily, since DNA markers can be assayed any time after a tissue or blood sample can be collected from the individual infant animal.

The use of genetic differences in receptor genes has become a valuable marker system for selection. For example, United States Patents 5,550,024 and 5,374,526 issued to Rothschild et al. disclose a polymorphism in the pig estrogen receptor gene which is associated with larger litter size, the disclosure of which is incorporated herein by reference. United States application serial number 08/812,208 discloses polymorphic markers in the pig prolactin receptor gene which are associated with larger litter size and overall reproductive efficiency.

It can be seen from the foregoing that a need exists for a method for selecting animals with the improved metabolic traits regarding fat content, growth rate, and feed consumption.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a genetic marker based on or within the MC4R gene which is indicative of fat content, growth rate, and/or feed consumption.

Another object of the invention is to provide an assay for determining the presence of this genetic marker.

A further object of the invention is to provide a method of evaluating animals that increases accuracy of selection and breeding methods for the desired traits.

Yet another object of the invention is to provide a PCR amplification test which will greatly expedite the determination of presence of the marker.

An additional object of the invention is to provide a kit for evaluating a sample of animal DNA for the identified genetic marker.

These and other objects, features, and advantages will become apparent after review of the following description and claims of the invention which follow.

This invention relates to the discovery of a polymorphism within the melanocortin-4 receptor (MC4R) gene which is associated with fat content, growth rate, and feed conversion traits in animals. According to the invention, the association of the MC4R polymorphism with the trait(s) enables genetic markers to be identified for specific breeds or genetic lines. The *TaqI* restriction pattern which identifies the polymorphism is used to assay for the presence or absence of markers associated with the desirable metabolic traits. The breed-dependent marker genotype (i.e., a marker in some breeds and a nonmarker in others) consists of a polymorphism within MC4R, a guanine to adenine transition at position 678 of the PCR product (a missense mutation of aspartic acid codon (GAU) into asparagine codon (AAU) at position 298 amino acid of the MC4R protein). The invention includes assays for detection of the marker as well as the sequence characterization of the polymorphism and includes novel sequences in the MC4R gene which may be used to design amplification primers for such an assay. Additionally, the invention includes a method for using the assay in breeding programs for animal selection and a kit for performing the assay.

Definitions

As used herein, "low fat content" or "leanness" means a biologically significant decrease in body fat relative to the mean of a given population.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the sequence listing for MC4R in pigs (SEQ ID NO:1). "X" represents the site of the polymorphism.

Figure 2 represents a comparison of the DNA sequence between the human (SEQ ID NO:2) and the porcine (SEQ ID NO:3) MC4R gene.

Figure 3 represents a comparison of the amino acid sequence between the human (SEQ ID NO:4) and the porcine (SEQ ID NO:5) MC4R gene.

Figures 4a, 4b, and 4c are linkage reports for MC4R from CRI-MAP.

Figure 5 depicts partial nucleotide and amino acid sequences (SEQ ID NO:12) of the porcine MC4R gene. The amino acid translation shows an amino acid substitution at codon 298.

Figure 6 is an electrophoresis gel of *TaqI* digestion of the PCR product. Molecular marker (M) and MC4R genotypes are indicated at the top of each lane.

Figure 7 depicts multiple-alignments of the putative seventh transmembrane domain of porcine MC4R with other MCRs and GPCRs. The "*" represents the predicted sequence positions for porcine MC4R. The other amino acid sequences were obtained from the GenBank database (accession numbers P32245, P70596, P41983, P56451, P34974, P41968, P33033, Q01718, Q01726, Q28031, AF011466, P21554, P18089, P30680, P47211). The missense variant in porcine MC4R substituted amino acid N for D in the position marked with an arrow. The Asp (D) residue is highly conserved among MCRs, and the Asn (N) residue is well conserved in most other GPCRs.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Obesity is a disease affecting energy balance. The control of energy metabolism is simple: store excessive energy as fat and manage the energy to avoid superfluous energy storage, i.e., obesity. Although several genes and signaling systems have been implicated in obesity, there has been little known about the interconnection of energy homeostatic mechanism and genetic polymorphism. The melanocortin-4 receptor (MC4R) has been shown to be an important mediator of long term weight homeostasis. MC4R antagonists can increase food intake and body weight during chronic administration. Skuladottir, G.V., et al., "Long term orexigenic effect of a novel melanocortin 4 receptor selective antagonist", British J. of Pharm., 126(1):27-34 (1999).

Lu et al. (1994) suggested that the melanocortin receptors are involved in controlling food intake and energy balance through studying its antagonism to the *agouti* obesity syndrome. Huszar et al. (1997) found that inactivation of the melanocortin-4

receptor gene (MC4R) resulted in a maturity onset obesity syndrome in mice and demonstrated a major role of MC4R protein in the regulation of energy balance related to the *agouti* obesity syndrome. In addition, the MC4R protein mediates the effects of leptin, one of the important signaling molecules in energy homeostasis (Seeley et al. 1997).

According to the present invention, a variant or polymorphism in the MC4R gene has been located, and this genetic variability is associated with phenotypic differences in the metabolic traits of fat content, growth rate, and/or feed consumption.

In one embodiment of the invention, an assay is provided for detection of presence of a desirable genotype. The assay involves amplifying the genomic DNA purified from blood, tissue, semen, or other convenient source of genetic material by the use of primers and standard techniques, such as the polymerase chain reaction (PCR), then digesting the DNA with a restriction enzyme (e.g., *Taq I*) so as to yield gene fragments of varying lengths, and separating at least some of the fragments from others (e.g., using electrophoresis).

The fragments may also be detected by hybridizing with a nucleotide probe (e.g., radio-labeled cDNA probes) that contains all or at least a portion of the MC4R gene cDNA sequence to the separated fragments and comparing the results of the hybridization with assay results for a gene sequence known to have the marker or a sequence known to not have the marker. Selection and use of probes for detection of MC4R sequences based on the known and disclosed MC4R sequences is generally known to those skilled in the art. The probe may be any sequence which will hybridize to the separated digestion products and allow for detection.

Another embodiment of the invention provides a kit for assaying the presence in a MC4R gene sequence of a genetic marker. The marker being indicative of inheritable traits of fat content, growth rate, and/or feed consumption. The kit in a preferred embodiment also includes novel PCR primers comprising 4-30 contiguous bases on either side of the polymorphism to provide an amplification system allowing for detection of the *Taq I* polymorphism by PCR and *Taq I* digestion of PCR products. The preferred primers are SEQ ID NO:8 and SEQ ID NO:9.

A further embodiment comprises a breeding method whereby an assay of the above type is conducted on a plurality of gene sequences from different animals or animal

embryos to be selected from and based on the results, certain animals are either selected or dropped out of the breeding program.

According to the invention, the polymorphism in the MC4R gene, identifiable by the *Taq I* restriction pattern, is disclosed. As is known in the art, restriction patterns are not exact determinants of the size of fragments and are only approximate. The polymorphism is identifiable by three bands from a *Taq I* digestion of the PCR product, 466, 225, and 76 base pairs (bp) for one homozygous genotype (allele 1); two bands, 542 and 225 bp for another homozygous genotype (allele 2); and four bands for the heterozygous genotype (542, 466, 225, and 76 bp). The marker for leanness and lower feed intake is identifiable by the 466/225/76 bands, except for the Chinese pigs, where the Chinese pigs' marker for leanness is the 542/225 bands. The marker for faster rate of gain is identifiable by the 542/225 bands.

In addition, the polymorphism associated with the pattern has been identified at the nucleotide level. The polymorphic *Taq I* site was sequenced along with the general surrounding area. See SEQ ID NO: 1. The sequences surrounding the polymorphism have facilitated the development of a PCR test in which a primer of about 4-30 contiguous bases taken from the sequence immediately adjacent to the polymorphism is used in connection with a polymerase chain reaction to greatly amplify the region before treatment with the *Taq I* restriction enzyme. The primers need not be the exact complement; substantially equivalent sequences are acceptable.

From sequence data, it was observed that in allele 2 the guanine is substituted with an adenine at position 678 of the PCR product or at position 298 amino acid of the MC4R protein changing the aspartic acid codon (GAU) into an asparagine codon (AAU). The PCR test for the polymorphism used a forward primer of 5'-TGG CAA TAG CCA AGA ACA AG-3' (SEQ. ID NO: 6) and a reverse primer of 5'-CAG GGG ATA GCA ACA GAT GA-3' (SEQ. ID NO: 7). Pig specific primers used were a forward primer of 5'-TTA AGT GGA GGA AGA AGG-3' (SEQ. ID NO: 8) and a reverse primer of 5'-CAT TAT GAC AGT TAA GCG G-3' (SEQ ID NO:9). The resulting amplified product of about 750 bp, when digested with *Taq I*, results in allelic fragments of 466, 225, and 76 bp (allele 1) or 542 and 225 bp (allele 2).

The marker may be identified by any method known to one of ordinary skill in the art which identifies the presence or absence of the marker, including for example, single-

strand conformation polymorphism analysis (SSCP), RFLP analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, and temperature gradient electrophoresis, ligase chain reaction or even direct sequencing of the MC4R gene and examination for the *Taq I* RFLP recognition pattern.

One or more additional restriction enzymes and/or probes and/or primers can be used. Additional enzymes, constructed probes, and primers can be determined by routine experimentation by those of ordinary skill in the art.

Other possible techniques include non-gel systems such as TaqMan™ (Perkin Elmer). In this system, oligonucleotide PCR primers are designed that flank the mutation in question and allow PCR amplification of the region. A third oligonucleotide probe is then designed to hybridize to the region containing the base subject to change between different alleles of the gene. This probe is labeled with fluorescent dyes at both the 5' and 3' ends. These dyes are chosen such that while in this proximity to each other the fluorescence of one of them is quenched by the other and cannot be detected. Extension by *Taq* DNA polymerase from the PCR primer positioned 5' on the template relative to the probe leads to the cleavage of the dye attached to the 5' end of the annealed probe through the 5' nuclease activity of the *Taq* DNA polymerase. This removes the quenching effect allowing detection of the fluorescence from the dye at the 3' end of the probe. The discrimination between different DNA sequences arises through the fact that if the hybridization of the probe to the template molecule is not complete, i.e., there is a mismatch of some form, the cleavage of the dye does not take place. Thus, only if the nucleotide sequence of the oligonucleotide probe is completely complementary to the template molecule to which it is bound will quenching be removed. A reaction mix can contain two different probe sequences each designed against different alleles that might be present, thus, allowing the detection of both alleles in on reaction.

Though the use of RFLPs is one method of detecting the polymorphism, other methods known to one of ordinary skill in the art may be used. Such methods include ones that analyze the polymorphic gene product and detect polymorphisms by detecting the resulting differences in the gene product.

Though the preferred method of separating restriction fragments is gel electrophoresis, other alternative methods known to one skilled in the art may be used to separate and determine the size of the restriction fragments.

It is possible to indirectly select for the polymorphism with alternative DNA markers. It is possible to establish a linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with the MC4R gene which have previously been shown to be associated with a particular trait. Examples of markers on the published PiGMaP chromosome map which are linked to the MC4R gene include S0331, BHT0433, and S0313.

The reagents suitable for applying the methods of the present invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. At a minimum, the kit contains a reagent that identifies the polymorphism in the MC4R gene that is associated with the traits of interest, fat content, growth rate, and feed consumption. Preferably, the reagent that identifies the polymorphism is a PCR set (a set of primers, DNA polymerase, and four nucleoside triphosphates) that hybridize with the MC4R gene or a fragment thereof. Preferably, the PCR set and restriction enzyme that cleaves the MC4R gene in at least one place are included in the kit. Preferably, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization, and similar purposes may also be included, if desired.

The genetic markers, methods, and kits of the invention are useful in a breeding program to improve fat content, growth rate, and feed consumption characteristics in a breed, line, or population of animals. Continuous selection and breeding of animals that are at least heterozygous and preferably homozygous for the desired polymorphism associated with the particular trait would lead to a breed, line, or population having those desired traits. Thus, the marker is a selection tool.

The following examples are offered to illustrate, but not limit the invention.

EXAMPLE 1

Melanocortin 4 Receptor PCR-RFLP Test - *TaqI* polymorphism and Genetic Linkage Mapping of MC4R Gene

Primers:

Primers were designed from homologous regions of human and rat MC4R sequences (Genbank Accession No. s77415 and u67863, respectively). These primers were used to amplify a 750-bp sequence of the porcine MC4R gene.

MC4R1: 5' TGG CAA TAG CCA AGA ACA AG 3' (SEQ ID NO:6)
MC4R4: 5' CAG GGG ATA GCA ACA GAT GA 3' (SEQ ID NO:7)

PCR Conditions:

Mix 1:	10X Promega Buffer	1.0 µL
	25 mM $MgCl_2$	0.6 µL
	dNTPs mix (2.5mM each)	0.5 µL
	25 pmol/µL MC4R1	0.1 µL
	25 pmol/µL MC4R4	0.1 µL
	dd sterile H_2O	7.5 µL
	Taq Polymerase (5 U/µL)	0.07 µL
	Genomic DNA (12.5 ng/µL)	1.0 µL

Ten µL of Mix 1 and DNA were combined in reaction tube, then overlaid with mineral oil. The following PCR program was run: 94°C for 2 min.; 35 cycles of 94°C for 30 sec.; 58°C 1 min., and 72°C 1 min. 30 sec.; followed by a final extension at 72°C for 15 min.

Five µl of the PCR reaction product was checked on a standard 1% agarose gel to confirm amplification success and clean negative control. Product size is approximately 750 base pairs. Digestion was performed by the following procedure.

*Taq*I Digestion Reaction	10 µL reaction
PCR product	5.0 µL
10X *Taq*I NE Buffer	1.0 µL
BSA (10mg/ml)	0.1 µL
*Taq*I enzyme (20 U/µL)	0.5 µL
dd sterile H_2O	3.4 µL

A cocktail of the buffer, enzyme, BSA, and water was made. Five μL was added to each reaction tube containing the DNA. The mixture was then incubated at 65°C for at least 4 hours to overnight. Loading dye was mixed with the digestion reaction and the total volume was loaded on a 3% agarose gel. The major bands for allele 1 are about 466, 225, and 76 bp. The allele 2 genotype bands are 542 and 225 bp. The heterozygote genotype has both allele 1 and allele 2.

Results

The amplified PCR product is about 750 bp. The sequence of the PCR product confirmed that the PCR product is MC4R gene with 97.6%, and 92.2% identities at the amino acid and DNA level, respectively, to corresponding human sequences. (see Figs. 2 and 3).

The *Taq*I digestion of the PCR product produced allelic fragments of 466, 225, and 76 bp (allele 1), or 542 and 225 bp (allele 2). The heterozygote genotype has both types of alleles. Mendelian inheritance was observed in three three-generation international reference families, which were used to map this gene by linkage analysis.

The polymorphism between allele 1 and allele 2 resulting from a G → A transition at position 678 of the PCR product revealed a missense mutation of Aspartic acid codon (GAU) into Asparagine codon (AAU) at position 298 amino acid of MC4R protein. (See Figure 1, SEQ ID NO:1).

Allele frequencies were determined by genotyping of DNA samples from a small number of animals from different breeds (Table 1). Allele 1 was observed with a frequency of 1 in Meishan, but was not observed or observed at very low frequency in Hampshire and Yorkshire. The frequencies of allele 1 in Landrace and Chester White were 0.5, respectively.

Figures 2 and 3 illustrate the differences between the DNA and amino acid sequences of the human and porcine MC4R gene (SEQ ID NOS:2-5).

TABLE 1

The Frequency of Allele 1 in Different Pig Breeds

Breed	# Animals	Freq. Allele 1
Meishan	8	1
Large White	8	0.56
Yorkshire	6	0.08
Hampshire	5	0
Landrace	4	0.5
Chester White	4	0.5
Minzu	2	1
Wild Boar	2	1

Linkage Analyses

Two-point and multi-point linkage analyses were performed on the genotypes of international reference families. See Figs. 4a-4c. The data were analyzed by using the CRI-MAP program. MC4R was significantly linked to several markers on porcine chromosome (SSC) 1. The most closely linked markers (recombination fraction and LOD score in parentheses) are SO331 (0.02, 21.97), BHT0433 (0.02, 21.32), and SO313 (0.00, 17.76) by two-point linkage analysis. A multi-point linkage analysis produced the best map order of markers and MC4R (with distance in Kosambi cM): KGF-5.8-CAPN3-2.5-MEF2A-6.1-MC4R-5.6-SO313.

Somatic cell hybrid panel of pig and rodent was used to assign MC4R to a cytogenetic region. PCR products from pig specific primers were amplified in clones 7, 8, 16, 18, and 19. MC4R was localized to SSClq 22-27.

EXAMPLE 2

MC4R Receptor PCR-RFLP Test using Pig Specific Primers and Genetic Linkage Mapping of the Porcine MC4R Gene

Pig Specific Primer Sequences

Forward primer: 5'-TTA AGT GGA GGA AGA AGG-3' (SEQ ID NO:8)

Reverse primer: 5'-CAT TAT GAC AGT TAA GCG G-3' (SEQ ID NO:9)

Method of Detection

The PCR reaction was performed using

Porcine genomic DNA	12.5 ng
1x PCR buffer	
$MgCl_2$	1.5 mM
dNTP	0.125 mM
Forward primer	0.3 μM
Reverse primer	0.3 μM
Taq DNA polymerase (Promega)	0.35 U

in a 10 μL final volume. The PCR profile included 2 min. at 94°C; 35 cycles of 30 sec. at 94°C, 1 min. at 56°C, 1 min. 30 sec. at 72°C; and 15 min. at 72°C in a Robocycler (Statagene, La Jolla, CA). A 5.0 μL aliquot of the PCR products was digested in a total volume of 10 μL with 10 U of *Taq*I incubated overnight at 65°C. The digestion products were electrophoresed on a 3% agarose gel.

Description of Polymorphism

The *Taq*I digestion of the PCR product produced fragments of 466, 225, and 76 bp in allele 1 and 542 and 225 bp in allele 2. The heterozygous genotype has fragments of both allele 1 and allele 2.

Pattern of Inheritance

Autosomal segregation of Mendelian inheritance was observed in three three-generation European PiGMaP families (Archibald et al., 1995).

Allele Frequencies

Allele frequencies were determined by genotyping the grandparental animals of the European PiGMaP families and unrelated animals from ISU reference families. Allele 1 was observed with the following frequencies.

TABLE 2

The Frequency of Allele 1 in Different Pig Breeds

Breed	# Animals	Freq. Allele 1
Meishan	8	1
Large White	8	0.56
Yorkshire	10	0.15
Hampshire	12	0
Landrace	8	0.56
Chester White	8	0.56
Minzu	2	1
Wild Boar	2	1

Chromosomal Location

Two-point and multi-point linkage analysis were performed on the genotypes of three PiGMaP families using the CRI-MAP program (Green et al. 1990). MC4R was significantly linked to several markers on porcine chromosome 1 (SSC 1). The most closely linked markers (recombination fraction and LOD score in parentheses) are SO331 (0.02, 21.97), BHT0433 (0.02, 21.32), and SO313 (0.00, 17.76) according to two-point linkage analysis. The best map order of MC4R with respect to other linked markers produced by multi-point linkage analysis is as follows (with distance in Kosambi cM): KGF-5.8-CAPN3-2.5-MEF2A-6.1-MC4R-5.6-SO313.

Comments

The Melanocortin-4 Receptor is a G protein-coupled, seven-transmembrane receptor expressed in the brain. Huszar et al. (1997) found that inactivation of MC4R gene resulted in a maturity onset obesity syndrome in mice and demonstrated a major role of MC4R protein in the regulation of energy balance. The MC4R gene has been mapped to human chromosome 18q21.3 (Gantz et al., 1993). The localization of MC4R gene to SSC 1 is consistent with previous chromosome painting data indicating synteny between this chromosome and HSA 18 and 15 (Goureau et al., 1996). However, the gene order of several genes previously mapped from HSA 18 and 15 to SSC 1, including CAPN3, KGF,

and MEF2A, is not conserved with MC4R. Therefore, mapping of MC4R to SSC 1 may identify an evolutionary breakpoint between HSA 18 and 15 in relation to SSC 1.

EXAMPLE 3

Association of Marker with Enhanced Metabolic Characteristics

In a preliminary study to determine which allele is associated with which trait and in which breeds, the genotypes of several lines of animals were correlated with days to 110 kg, backfat measurements, daily gains, and average daily feed intake. The pigs used in the study were from lines from Pig Improvement Company (PIC).

Data was accumulated using the PCR test described *supra* for the 1 and 2 allele of the MC4R gene. The collected data is summarized in Tables 3-8 below.

According to the results, allele 1 is the significantly leaner allele (see P2 backfat measurements) in all lines except in Chinese pigs where it is the fat allele. Allele 2 is associated with significantly faster rate of gain (test daily gain) in the tested commercial lines. Overall allele 1 is associated with lower feed intake.

TABLE 3

Number of observations

MC4R genotype	L02	L03	L19	L65	Overall	L95
11	88	30		32	150	20
12	57	54	56	74	241	67
22	12	31	254	33	330	37
				Total	721	

MC4R genotype:

11 = homozygous allele 1

12 = heterozygous

22 = homozygous allele 2

TABLE 4

Number of observations (males/females)

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	9/79	12/18		15/17	36/114		0/20
12	9/48	37/17	12/44	44/30	102/139		0/67
22	3/9	28/3	89/165	21/12	141/189		0/37

TABLE 5

Days to 110kg

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	169.7	172.4		169.6	168.5		219.1
12	170.2	171.5	165.0	171.2	168.7		212.2
22	165.3	173.4	162.9	170.3	167.1		211.4
	P<.23	P<.75	P<.15	P<.76	P<.31		P<.27

TABLE 6

P2 backfat (mm)

MC4R Genotype	L02	L03	L19	L65	Overall		L95
11	10.8	11.9		9.7	11.1		22.8
12	11.3	12.5	12.2	10.5	11.8		21.5
22	12.1	12.7	12.6	10.7	12.1		20.3
	P<.10	P<.43	P<.34	P<.17	P<.006		P<.17

TABLE 7

Test daily gain (gm/d)

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	882.2	811.0		881.8	871.9		688.8
12	891.2	820.5	875.6	873.0	876.3		676.2
22	969.1	819.5	906.7	906.2	906.9		692.5
	P<.01	P<.96	P<.05	P<.24	P<.006		P<.66

TABLE 8

Average daily feed intake (kg/d), boars only, except L95 which was gilts only

MC4R genotype	L02	L03	L19	L65	Overall		L95
11	2.31	1.78		1.75	1.89		2.05
12	2.11	1.90	1.97	1.90	1.96		2.03
22	2.15	1.97	2.00	1.97	2.02		2.08
	P<.84	P<.14	P<.56	P<.14	P<.16		P<.36

EXAMPLE 4

A Missense Variant of the Porcine Melanocortin-4 Receptor (*MC4R*) Gene is Associated with Fatness, Growth, and Feed Intake Traits

To determine if there was an association of this *MC4R* polymorphism with phenotypic variation the mutation was tested in a large number of individual animals from several different pig lines. Analyses of growth and performance test records showed significant associations of *MC4R* genotypes with backfat, growth rate and feed intake in a number of lines. It is probable that the variant amino acid residue of the *MC4R* mutation causes a significant change of the MC4R function. These results support the functional significance of a pig *MC4R* missense mutation and suggest that comparative genomics

based on model species may be equally important for application to farm animals as they are for human medicine.

Identification of mutations in the *leptin* and the *leptin receptor* has provided some information on genetic components involved in the regulation of energy balance (Zhang et al. 1994; Tartaglia et al. 1995). Genetic studies using animal models have facilitated the identification of major genetic causes of obesity (Andersson 1996; Pomp 1997; Giridharan 1998). Furthermore, several other genes involved in the neural signaling pathway of energy homeostasis have been identified (Flier and Maratos-Flier 1998; Schwartz et al. 1999). Of particular interest among candidate signaling molecules involved in the regulation of energy homeostasis is the melanocortin-4 receptor (MC4R). The MC4R response to leptin signaling is a link between food intake and body weight (Seeley et al. 1997; Marsh et al. 1999). Neuropeptide Y (NPY) signaling in the central nervous system is also mediated by the MC4R protein (Kask et al. 1998). Several mutations in MC4R including frameshift and nonsense mutations are associated with dominantly inherited obesity in humans (Vaisse et al. 1998; Yeo et al. 1998). Some other MC4R missense mutations in humans have also been identified (Gotoda et al. 1997; Hinney et al. 1999) but the functional significance of these mutations has not been characterized.

Selection based on growth characteristics has been of great importance to the pig industry because of costs associated with feeding and consumer preference for lean meat. Efficient genetic improvement in these quantitative traits may be augmented through the use of marker assisted selection (MAS) using high density genetic maps (Dekkers and van Arendonk 1998; Rothschild and Plastow 1999). An important tool in this process is comparative mapping using the well-developed human and mouse gene maps, which assist in the identification of corresponding genomic regions or major genes controlling growth and performance traits in the pig. Biological understanding of complex traits in human or model species offers an alternative approach to identify genes responsible for the traits of economic interest in livestock. Several quantitative trait loci (QTL) linkage scans using phenotypically divergent breeds and candidate gene analyses have been successfully conducted for fatness and growth traits (Yu et al. 1995; Casas-Carrillo et al. 1997; Knorr et al. 1997; Knott et al. 1998; Rohrer et al. 1998; Wang et al. 1998; Paszek et al. 1999), but no individual genes with major effects on growth and performance traits have yet been

established for commercial populations. The role of MC4R in feed intake and obesity suggests it may be an important genetic marker for the growth-related traits in the pig.

Materials and Methods

Animals. Pigs were raised under normal production conditions under the care of PIC employees in nucleus farms in the United States and Europe. Pigs were put on the performance test at approximately 70 days of age and taken off test after 13 weeks. At the end of the trial backfat was measured ultrasonically in real time (B mode) at the 10^{th} rib 2 cm from the centerline. Average daily gain (growth) over the test period was calculated as weight gained divided by days on test. Days to 110 kg market weight was estimated using standard procedures and feed intake was measured using individual electronic measurement equipment.

PCR amplification of a pig MC4R gene fragment. Primers were designed from homologous regions of human and rat MC4R sequences (GenBank accession no. s77415 and u67863, respectively). The primers were: forward primer: 5'-TGG CAA TAG CCA AGA ACA AG-3' (SEQ. ID NO:6) and reverse primer: 5'-CAG GGG ATA GCA ACA GAT GA-3' (SEQ. ID NO:7). The PCR reaction was performed using 12.5 ng of porcine genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.3 mM of each primer, and 0.35 U *Taq* DNA polymerase (Promega) in a 10μL final volume. The conditions for PCR were as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at 56°C, 1 min 30 s at 92°C, and a final 15 min extension at 72°C in a Robocycler (Stratagene, La Jolla, CA).

Sequencing and mutation detection. Sequencing of the PCR products from several individual pigs of different breeds was conducted and the sequences were compared to detect any nucleotide change. Sequencing was performed on an ABI sequencer 377 (Applied Biosystems). The porcine MC4R sequence has been submitted to GenBank, and has accession number AF087937. The sequence analysis revealed one nucleotide substitution situated within a *TaqI* restriction enzyme recognition site (Kim et al. 1999). A set of primers was then designed to generate a smaller MC4R gene fragment, which contained only one informative *TaqI* restriction site to specify the polymorphic site and to facilitate the PCR-RFLP test. These primers were: forward 5'-TAC CCT GAC CAT CTT

GAT TG-3' (SEQ. ID NO:10) and reverse: 5'-ATA GCA ACA GAT GAT CTC TTT G-3' (SEQ. ID NO:11).

Statistical analysis. Analysis of variance procedures were used with a mixed model that accounted for the fixed effects of farm, test period, sex of the animal, the MC4R genotype and site (random). All animals in lines of American/European descent (Lines A-D) were pooled for the overall analysis and in this analysis line of origin was included. Mean effects were estimated for each genotype and are presented in Tables 9-15. Overall F tests were used to determine level of significance.

Results

Identification of a missense mutation in the pig MC4R gene. The MC4R gene consists of approximately 1 kb of coding sequence contained within a single exon. About 750 bp of a pig MC4R gene fragment was produced by PCR (Kim et al. 1999). The sequence of the PCR product confirmed that the PCR product is the MC4R gene with 92.2% and 97.6% identities at nucleotide and the amino acid levels, respectively, to the human MC4R sequence. Multiple alignments of the sequences from individual animals of several breeds identified a single nucleotide substitution (G→A; Fig. 5). The polymorphism revealed a missense mutation that replaces aspartic acid (GAU) with asparagine (AAU) at the position identical to amino acid 298 of human MC4R protein. To confirm this base change, we designed pig-specific primers flanking the polymorphic site and analyzed the polymorphism as a *TaqI* PCR-RFLP gel (Fig. 6). Figure 6 shows a *TaqI* digestion of the PCR product analyzed by agarose-gel electrophoresis. Allele 1 produced 156 and 70 bp fragments and allele 2 produced a 226 bp fragment as the PCR-RFLP. The heterozygote has both allele 1 and 2 fragments. Molecular marker (M) and MC4R genotypes are indicated at the top of each lane.

The MC4R missense mutation is within a highly conserved region among melanocortin receptors (MCR). The MCR is a subfamily of G-protein coupled receptors (GPCR) containing certain conserved structural elements common to most other GPCRs, but overall amino acid identities between MCR and other GPCRs are low (Tatro 1996). A multiple-alignment of the predicted amino acid sequences of the pig MC4R with MC4R

proteins from other species, other MCR proteins, or representative GPCRs showed that the aspartic acid found at position 298 of the seventh transmembrane domain is very highly conserved in the MCR proteins (Fig. 7). It is interesting to note, however, that this position is occupied by asparagine in most other GPCRs. The MCR proteins show 40-80% amino acid identity with each other (Tatro 1996), but the second intracytoplasmic loop and the seventh transmembrane domain are highly conserved among MCR proteins (Gantz et al. 1993). Some of the relationships between MCR structure and function have been discovered by the studies of natural and experimental mutations in humans and mice (Robbins et al. 1993; Valverde et al. 1995; Frandberg et al. 1998). These studies indicate that some mutations in highly conserved regions cause structural changes and alter the function of the receptor. The Asp298Asn substitution mutation could have an effect on the function of the receptor. However, this will require further testing but it is known that change of the homologous residue in MC1R (Asp294His) is associated with fair skin and red hair in humans (Valverde et al. 1995).

The MC4R missense mutation is associated with obesity-related traits. To investigate the effects of the missense mutation, the relationship of MC4R genotypes was analyzed for the effects on variation in growth rate, backfat, and feed consumed in over 1,800 animals from several commercial pig lines from PIC, an international pig breeding company. The animals were from closed commercial lines of European/American breeds (Lines A-D) together with a line originating from a cross between a European and a Chinese breed (Line E). In lines A-D significant associations of the MC4R genotypes were found for all performance traits. The animals homozygous for allele 1 had on average significantly less backfat ($P < .001$), lower daily gain ($P < .001$), and lower feed intake($P < .01$) than those of the homozygous 22 genotype animals (Tables 11, 13, & 15). Overall, pigs with the 11 genotype had approximately 9% less backfat than pigs with the 22 genotype (Table 11), whereas pigs with the 22 genotype grow significantly faster (37g/day) than pigs with the 11 genotype (Table 13). These results appear to be a function of appetite because the 22 genotype animals consume considerably more feed (Table 15). The association between the missense variant of the MC4R gene and related performance traits is clearly established in European/American breeds. Although the number of tested animals is much smaller, these results were not seen in the considerably fatter Chinese

crossed line (Line E). Interestingly, line E shows a trend for backfat in the opposite direction to that observed in the other lines (Table 11).

Discussion

The present study clearly demonstrates that the porcine MC4R missense mutation is significantly associated with several performance traits in pigs. Allele 1 representing Asp298, the well conserved amino acid within other MCR subtypes and other species MC4R, was associated with less backfat thickness, slower growth rate, and lower feed intake and allele 2 representing Asn298 was associated with fatter, higher feed intake, and faster growing animals. As the highly conserved residues in the melanocortin receptor proteins have important roles for ligand binding or intracellular signal transmission (Tatro 1996), the MC4R variants might exert functionally distinct abilities in the regulation of food intake and body weight. Further testing of this hypothesis will provide important insights into the structural basis of MCR function and a molecular target for the treatment of human obesity.

Allele 1 was associated with the fattest animals in Line E, which was derived by crossing a Chinese Large White breed with a line of Meishan origin. This is surprising given that the mutation causes a significant amino acid change in a well-conserved region. The result may be due to sampling. However, if we assume that this result will be significant when more results are added there are several possible explanations. One possibility could be the difference in the background gene effects (epistasis). As growth and fatness are complex polygenic traits, it is certainly possible that the Chinese breed has some distinct allelic interactions derived from several hundred years of isolation and these putative interaction(s) might create variation in polygenic traits within crosses between widely different lines (Frankel and Schork 1996). Several QTL analyses have been conducted for fatness and growth traits using divergent lines (Cases-Carrillo et al. 1997; Knott et al. 1998; Rohrer et al. 1998; Wang et al. 1998; Paszek et al. 1999), but QTL have not been reported near the C4R locus, which maps to chromosome 1 at approximately 80 cm on the linkage map (data not shown). It may mean that the epistatic effects of the MC4R alleles suggested in Line E have made it difficult to observe the MC4R locus in most QTL experiments which have involved crosses between Chinese and European/American lines. It is likely that the effect of some alleles will be variable in the

different backgrounds and hard to detect in QTL experiments involving genetically divergent breeds.

The effect of MC4R variant will possibly be explained by further studies on the biological effect caused by this mutation in other pig breeds and lines. However, given the strong relationship of MC4R variants to leanness, growth and feed intake, this mutation could be used immediately for merker assisted selection (Meuwissen and Goddard 1996) to develop lines of pigs to satisfy particular customer requirements. For instance, in sow lines where appetite is normally decreased after farrowing, selection for the MC4R 2 allele could help improve feed intake. Furthermore, in some lines deemed to be too fat, selection for allele 1 could be employed and in lines that were considered too slow growth allele 2 selection could be also employed. Therefore, genotyping for the MC4R mutation in pig breeding lines will improve the selection efficiency of feed related production traits including growth and leanness. The candidate gene approach has also been used for investigating the role of the porcine leptin gene (Jiang and Gibson 1999). However, in the leptin case, although there was evidence for an association between a leptin polymorphism and backfat depth in a cross between a commercial breed and an unimproved line, there was no clear association in the different commercial lines tested (Jiang and Gibson 1999). Therefore, it should not be assumed that since one finds a gene that one can assume a relationship exists. In contrast, with MC4R we have determined that variation in this candidate gene can explain significant variation for backfat, growth rate, and feed intake in commercial lines of pigs. These results with MC4R illustrate the potential value of comparative genetic analyses using candidate genes in livestock genomics.

EFFECT OF MC4R GENOTYPE ON SEVERAL PRODUCTION TRAITS IN THE PIG

TABLE 9

Number of observations (males/females/totals) for Days to 110 kg and backfat

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	9/212/221	12/94/106		37/17/54	58/323/381	0/20/20
12	9/150/159	37/96/133	12/158/170	152/30/182	210/434/644	0/67/67
22	3/16/19	28/36/64	89/356/445	155/12/167	275/420/695	0/37/37

TABLE 10

Days to 110kg

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	166.3+/-0.8	168.4+/-1.4		170.0+/-2.4	167.9+/-0.9	219.1+/-4.8
12	165.6+/-0.9	166.8+/-1.1	163.9+/-1.0	170.2+/-1.8	166.9+/-0.8	212.2+/-3.4
22	162.3+/-2.3	166.8+/-1.5	161.5+/-0.8	167.0+/-1.9	164.6+/-0.9	211.4+/-4.0
	P <.24	P <.47	P <.007	P <.10	P <.001	P <.27

TABLE 11

10^{th} rib Backfat (mm)

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	10.7+/-0.2	12.1+/-0.2		9.8+/-0.5	11.1+/-0.2	22.8+/-1.2
12	11.2+/-0.2	12.5+/-0.2	12.3+/-0.2	10.5+/-0.4	11.6+/-0.2	21.5+/-0.9
22	12.5+/-0.5	12.6+/-0.3	12.7+/-0.2	10.9+/-0.4	12.0+/-0.2	20.3+/-1.0
	P <.02	P <.31	P <.06	P <.05	P <.001	P <.17

TABLE 12

Number of observations (males/females/totals for Test daily gain

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	9/105/114	12/38/50		37/17/54	58/160/218	0/20/20
12	9/65/74	37/35/72	12/97/109	152/30/182	210/227/437	0/67/67
22	3/13/15	28/15/43	89/225/314	155/12/167	275/265/539	0/37/37

TABLE 13

Test daily gain (gm/day)

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	892.6+/-10.4	841.7+/-13.8		882.2+/-18.4	871.9+/-10.2	688.8+/-24.5
12	913.3+/-11.6	868.4+/-12.1	882.2+/-12.9	883.7+/-14.3	885.1+/-8.9	676.2+/-17.6
22	982.8+/-22.8	862.4+/-15.1	913.4+/-10.5	904.6+/-15.1	908.8+/-9.3	692.5+/-20.4
	P <.001	P <.28	P <.006	P <.20	P <.001	P <.66

TABLE 14

Number of observations (males/females/total) for average daily feed intake

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	7/0/7	11/0/11		13/0/13	31/0/31	0/18/18
12	8/0/8	31/0/31	9/0/9	34/0/34	82/0/82	0/63/63
22	3/0/3	25/0/25	74/0/74	16/0/16	118/0/118	0/32/32

TABLE 15

Average daily feed intake (kg/day), boars only except LINE E which was gilts only

MC4R	LINE A	LINE B	LINE C	LINE D	Total	Line E
11	2.31+/-0.2	1.78+/-0.09		1.75+/-0.06	1.94+/-0.07	2.05+/-0.10
12	2.11+/-0.3	1.90+/-0.07	1.97+/-0.10	1.90+/-0.07	2.03+/-0.06	2.03+/-0.07
22	2.15+/-0.4	1.97+/-0.06	2.00+/-0.07	1.97+/-0.08	2.11+/-0.06	2.08+/-0.08
	P <.84	P <.14	P <.56	P <.14	P <.01	P <.36

Having described the invention with reference to particular compositions, theories of effectiveness, and the like, it will be apparent to those of skill in the art that it is not intended that the invention be limited by such illustrative embodiments or mechanisms, and that modifications can be made without departing from the scope or spirit of the invention, as defined by the appended claims. It is intended that all such obvious modifications and variations be included within the scope of the present invention as

defined in the appended claims. The claims are meant to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates to the contrary.

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